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ORIGINAL ARTICLE

A Novel Technique for Detecting the Therapeutic Target, *KRAS* Mutant, From Peripheral Blood Using the Automatic Chipball Device With Weighted Enzymatic Chip Array

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Reverse transcriptase and real-time polymerase chain reactions are widely used for the detection of gene overexpression. However, various disadvantages and limitations arise when the detection of multiple genetic targets is required. In previous studies, our laboratory successfully established a membrane array operation platform with a diagnostic biochip for the screening of gene overexpression by circulating tumor cells in cancer patients. To effectively shorten the reaction time, we improved the conventional RNA extraction method. The concept of weightedness was included in the reading procedure of the chip array and a weighted enzymatic chip array (WEnCA) platform was established. We used fluid engineering to develop a fully automatic gene chip analyzer named Chipball, which runs automatically on the WEnCA platform. The combination of the two systems is named the WEnCA-Chipball system. To understand the actual differences between the operations of WEnCA-Chipball and WEnCA-manual, we used the WEnCA-manual to analyze *KRAS*-associated gene overexpression in 200 samples from cancer patients to establish a cutoff value for activating the *KRAS* Detection Chip. Specifically, the activated *KRAS* expression in blood samples of 209 lung cancer patients was analyzed by both WEnCA-manual and WEnCA-Chipball and compared. The clinical applicability of WEnCA-Chipball was defined, including the sensitivity, specificity, and accuracy. The results showed that among 209 samples, 71 patients were positive for activated *KRAS* expression by WEnCA-Chipball with a sensitivity of 89%, specificity of 94%, and accuracy of 92%. In addition, the average total score of WEnCA-Chipball was 4.7 lower than that of the WEnCA-manual. The WEnCA-Chipball required an operation time of only 7.5 hours, approximately one-ninth of the

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WEnCA-manual operation time and one-fifth of the cost of WEnCA-manual. No significant difference was found between the detection limitations of the two systems. Great strides have been made in this development. The WEnCA-Chipball operation system has potential for clinical applications.

Key Words: Activating KRAS Detection Chip; WEnCA-manual; WEnCA-Chipball

Introduction

The analysis of gene overexpression has led to fundamental progress and clinical advances in the diagnosis of disease.[1,2] The techniques that are commonly used to study gene overexpression include Northern blotting, and reverse transcriptase (RT) and real-time polymerase chain reactions (PCRs).[3–5] Since Northern blotting involves complex steps and a large numbers of samples, its application is limited to research instead of clinical diagnosis. On the other hand, since RT-PCR and real-time PCR are performed through a series of simple steps, they are applied extensively for the detection of single genes, including the hepatitis virus and other infectious pathogens.[6,7] Although the invention of PCR ranks as one of the great scientific inventions, most PCR techniques have a few common problems: (1) contamination, such as false-positive results from oversensitive detection of, say, aerosolized DNA or previous sample carry-over; (2) RT-PCR is regarded as only semi-quantitative, since it is difficult to control the efficiency of sequence amplification when comparing different samples; and (3) interference is caused by annealing between the primers. RT-PCR or real-time PCR is used extensively in the detection of a single-gene target.[8–10] For the detection of multiple targets or gene clusters, PCR-related techniques tend to be time-consuming, cumbersome, and costly.

The rapid development of biotechnology in recent years has made biochips an important tool in clinical diagnosis or drug efficacy evaluation.[11] Our previous study has developed and evaluated a membrane array-based method for simultaneously detecting the expression levels of multiple messenger RNA (mRNA) markers from circulating cancer cells in the peripheral blood for cancer diagnosis.[12] In those studies, the expression levels of molecular markers were simultaneously evaluated by RT-PCR and membrane array. Data obtained from RT-PCR and membrane array were subjected to linear regression analysis, revealing a high degree of correlation between the results of these two methods ($r=0.979$, $p<0.0001$).[13]

The application of the membrane array for cancer diagnosis has been successfully proven in our previously studies. However, at the time of outcome reading, every gene is calculated by the same value; this does not differentiate the importance of each gene for specific disease diagnoses, a major limitation of this technique in clinical application.[14] For this study, the cost of the digoxigenin enzyme used on the colorimetric biochip platform was too high for laboratory diagnosis, and the high criteria of the operation techniques prevented its widespread availability for clinical applications. Therefore, we developed the next generation biochip operation platform (weighted enzymatic chip array [WEnCA]). The technical difference between the WEnCA system and the conventional membrane array includes the different weighted multiples for each gene target on the biochip, dependent on the importance of each gene during the cancer development process. Furthermore, the conventional digoxigenin system was replaced by the biotin-avidin enzyme system to lower the cost (Figure 1). The manual operation platform used has been successfully established and published;[15,16] the aim of the current study was to further establish an automatic system of WEnCA-Chipball by incorporating recently developed fluid engineering (Figure 2). As a result, the WEnCA platform may be automatically operated to effectively reduce the manual errors and limitations due to technical criteria.

With the rapid advancements in the field of fluid engineering, and especially biomedicine in recent years, automated and rapid biomedical analysis is now considered to offer the greatest potential and market value.[17,18] In terms of biomedical development, the automated fluid biomedical test system that was produced through fluid engineering has the advantages of high detection sensitivity, portability, low sample/test sample consumption, low power consumption, small size, and low cost. Compared with conventional analysis techniques, it represents a significant breakthrough. With a variety of innovative techniques, a wide range of precision fluid components have been manufactured to control biological fluids, reduce the size of the biochemical analytical instruments, and integrate the processes into a one-step automated system that facilitates the rapid conducting of biomedical tests.[11] In this experiment, the fluid engineering technique was adopted to automate the WEnCA platform, significantly reduce detection time, and reduce errors

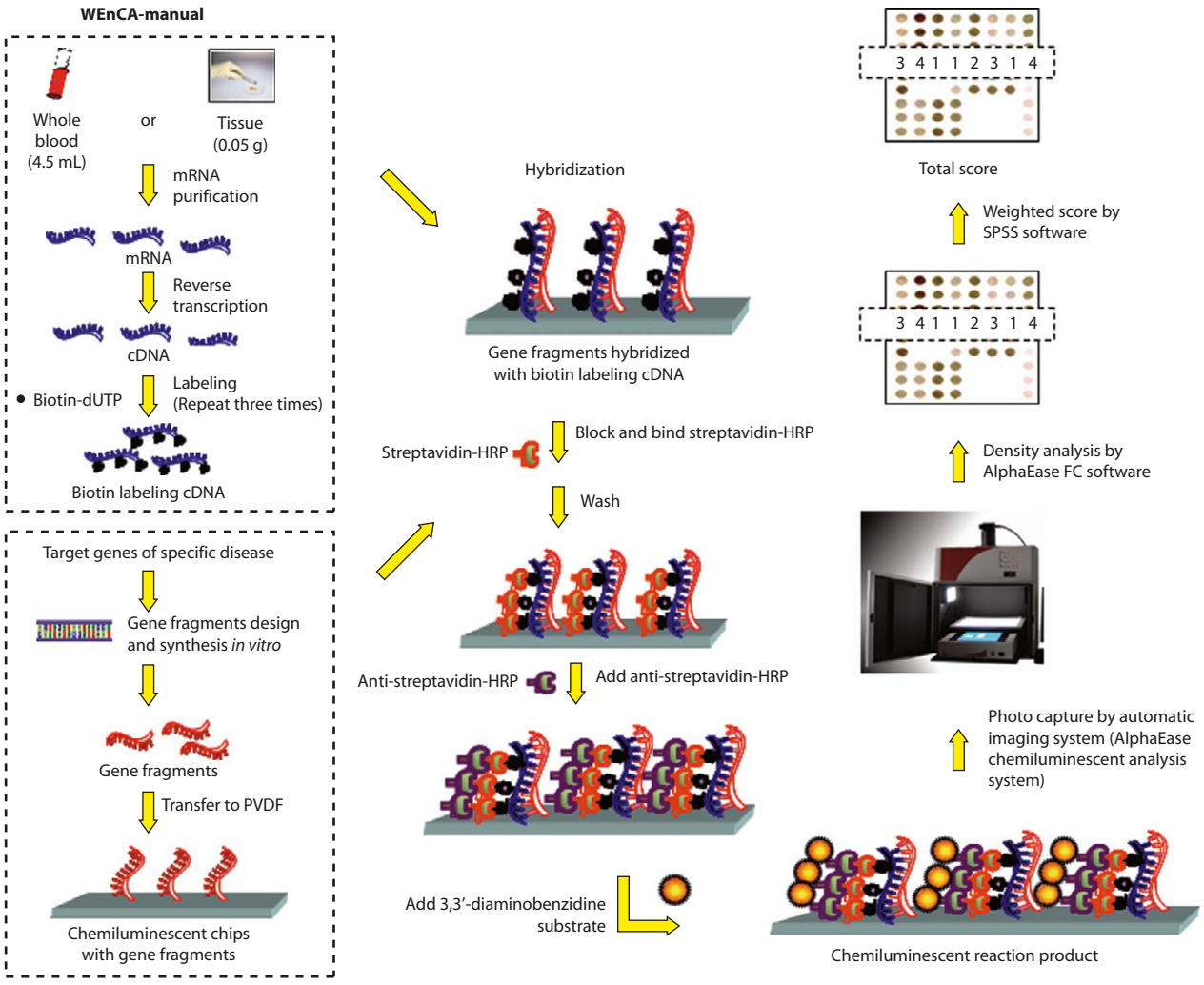


Figure 1 Manual operation platform of the weighted enzymatic chip array (WEnCA). dUTP: deoxyuridine triphosphate; HRP: horseradish peroxidase; PVDF: polyvinylidene difluoride.

arising from human operation. Thus, the bottleneck that was preventing the commercialization of the chip detection technique has been overcome.

To understand the clinical applicability of WEnCA-Chipball, and the resulting differences between the actual operations of WEnCA-Chipball and WEnCA-manual, we first analyzed *KRAS* associated gene expression using the WEnCA-manual to establish the cutoff value for the Activating *KRAS* Detection Chip. Next, the activated *KRAS* expression in blood samples of 209 lung cancer patients was determined according to the experimental procedure shown in Figure 3. They were then analyzed by both WEnCA-manual and WEnCA-Chipball; the results were compared and the clinical applicability of WEnCA-Chipball was defined. Further comparisons were performed on the sensitivity, the specificity, and the accuracy of the WEnCA manual and automatic operation platform. The application, operation time, and cost of the two platforms were also investigated to evaluate the clinical application potential of WEnCA-Chipball.

Materials and Methods

Specimen collection

Initially, cancer tissues were collected from 200 selected cancer patients enrolled in this study, including 85 patients with breast cancer, 64 patients with colorectal cancer (CRC), and 51 patients with non-small cell lung cancer (NSCLC) who had undergone surgical resection or biopsy between January 2007 and December 2008. The data from the 200 cancerous patients were used for the analysis of sensitivity, specificity, and diagnostic accuracy of WEnCA. Tissue samples from various cancer patients were divided into two groups: one group of 100 cancer tissues with the *KRAS* mutation (including

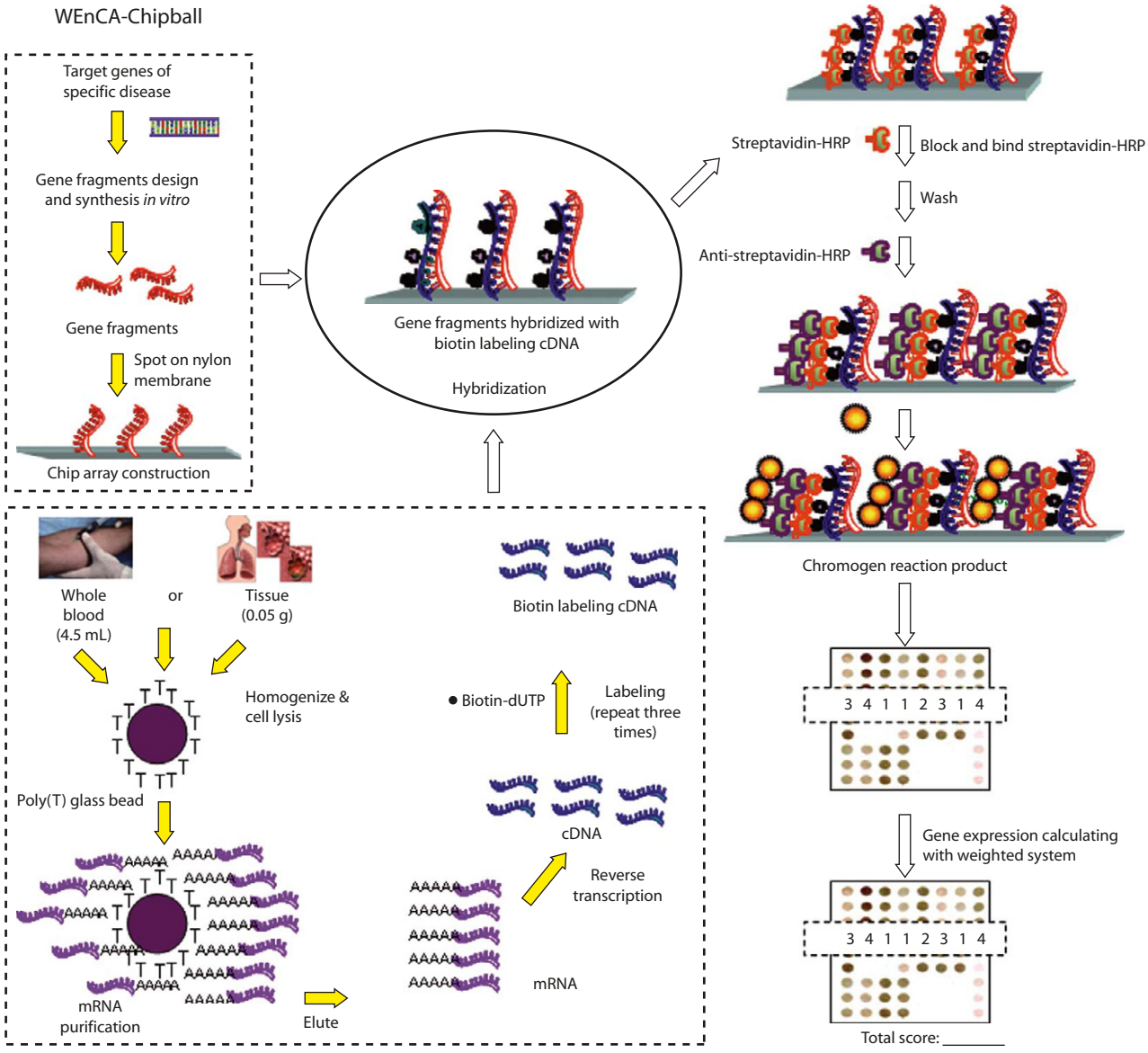


Figure 2 The automatic Weighted Enzymatic Chip array (WEnCA)-Chipball operation platform. dUTP: deoxyuridine triphosphate; HRP: horseradish peroxidase.

32 CRCs, 51 breast cancers, and 17 NSCLCs), and one group of 100 cancer tissues without the *KRAS* mutation (including 32 CRCs, 34 breast cancers and 34 NSCLCs). These groups were used to determine the cutoff value of the WEnCA method for further circulating tumor cell analysis of 209 lung cancer patients. To clinically evaluate and compare both two systems, WEnCA-manual and WEnCA-Chipball, blood specimens from 209 lung cancer patients were collected within test tubes containing anticoagulant sodium citrate. To avoid contamination of skin cells, the sampled blood was taken via an intravenous catheter, plus the first few milliliters of blood were discarded. Total RNA was immediately extracted from the peripheral whole blood, and then served as a template for complementary DNA (cDNA) synthesis. Sample acquisition and subsequent usage were approved by the institutional review boards of three hospitals. Written informed consent was obtained from all participants.

WEnCA-manual

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the collected cancer tissue specimens using the acid-guanidinium-phenol-chloroform method according to standard protocol.[19] The RNA concentration was determined spectrophotometrically based on the absorbance at 260 nm. First-strand cDNA was synthesized from total RNA using the Advantage RT-PCR kit (Promega, Madison, WI, USA) and reverse transcription was performed in a reaction mixture consisting of transcription optimized

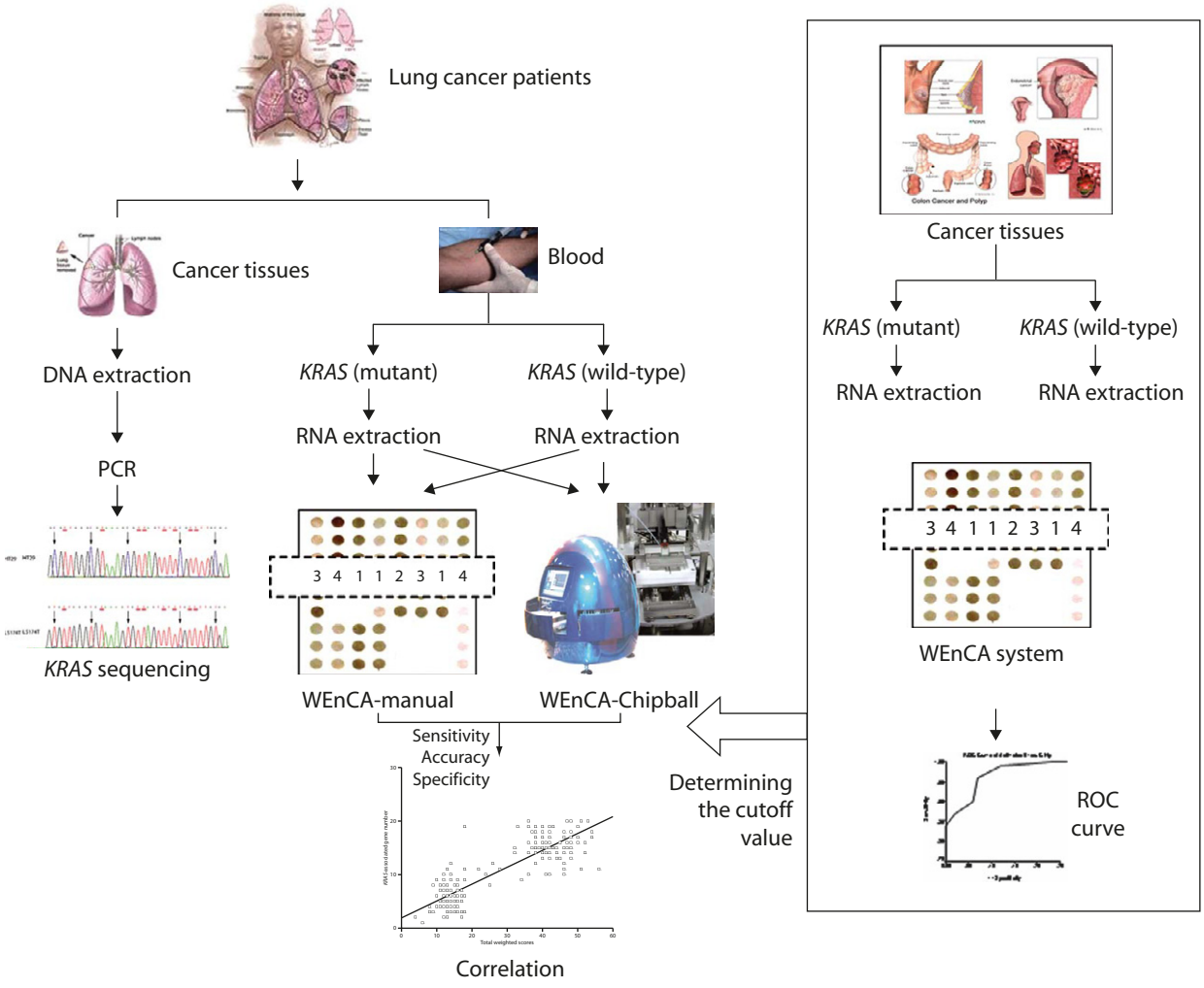


Figure 3 Study flowchart.

buffer, oligo(dT)$_{15}$ primer at 25 mg/mL 100mM PCR nucleotide mix, 200mM Moloney murine leukemia virus reverse transcriptase, and 25 mL of recombinant ribonuclease inhibitor. The reaction mixtures were incubated at 42°C for 2 hours, heated to 95°C for 5 minutes, and then stored at 48°C until analysis.

Weighted enzymatic chip array

The oligo chip preparation was the same as previously described.[2,4,6,13] The labeling of the first cDNA and operating procedures for chip hybridization were also the same as previously described; however, the probe labeling in this study used biotin in place of digoxigenin. After hybridization, the chips were washed, blocked, and then incubated for 30 minutes in horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada), diluted in TRIS buffered saline. The blot was processed using the 3,3′-diaminobenzidine kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The colorimetric signal was detected with an Alpha Innotech Image system (Alpha Innotech Corp., San Leandro, CA, USA), and the intensity of each spot on the membrane was measured using AlphaEase FC software (Alpha Innotech Corp). The criteria for determining the overexpressed spot are described elsewhere.[2,4,6,13] Each positive spot was multiplied by different weighted values ranging from 1 to 4 based on the performance after *KRAS* activation to calculate the total score of the chip.

WEnCA-Chipball system

mRNA extraction

Biotin poly(dT) and streptavidin magnetic beads were used to isolate the mRNA. First, the cells were disintegrated by adding lysis buffer to the blood samples. Magnetic glass particles were then added to combine with the RNA in the cells and

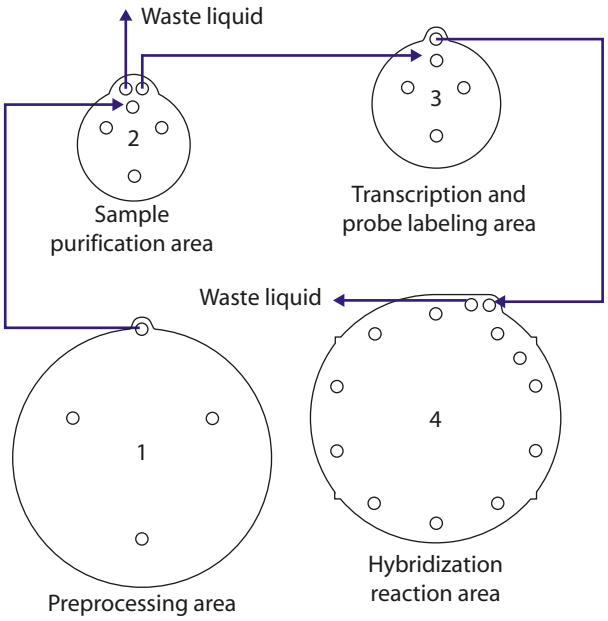


Figure 4 Automatic microfluid reaction chamber design.

flush/separate the nucleic acids on the magnetic beads and buffer. After that, the mRNA was eluted and stored for later use.

Chipball operating procedure

First, to breakdown the blood cells and isolate mRNA, the fluid delivery control system delivers blood and lysis buffer to the first reaction chamber (sample preprocessing area) (Figure 4) through the fluid input area. The fluid delivery control system also delivers the ChargeSwitch Lysis Magnetic Beads (Invitrogen Corp., Carlsbad, CA, USA), binding buffer, and wash buffer through the fluid input area to the first chamber. The samples are mixed (eddy effect) by the spin tray on the active mixer device to ensure that the samples react effectively enough for the RNA to combine with the magnetic beads by means of an electric charge connection. The reacted samples and the beads that have absorbed mRNA onto the surface can then be delivered to the second reaction chamber (sample purification area) by the fluid delivery control system. Magnetic beads are used to separate and purify the targeted samples in this area. The reacted magnetic beads that have the samples absorbed onto their surface can be collected by the magnetism control system and transmitted to the wash buffer by the fluid delivery control system. The remaining waste liquid, except the beads containing mRNA, can be emitted by the fluid delivery control system through the waste collection area. The beads with absorbed RNA are then demagnetized, and the waste collection area is closed. The elution buffer is delivered through the fluid delivery control system to the reaction area for the mixing reactions. The purified mRNA samples that have been extracted are then delivered through the fluid delivery control system and the fluid propellant area to the third reaction chamber (transcription), and then to the probe labeling area. The purified mRNA samples are then delivered to the reaction chamber for mixing reactions through the fluid delivery control system. The reaction temperature needed by the transcription reaction can be regulated by the temperature control system, allowing the mRNA to be converted into stable cDNA for chromogen labeling on the biomolecular test target. The reacted samples are then delivered by the fluid delivery control system through the buffer to the hybridization and color development area for the hybridization reaction. Meanwhile, before delivering the samples to the fourth reaction chamber (hybridization reaction area), the gene chips are placed in the hybridization and color development area for the prehybridization reaction. The labeled cDNA samples then enter the fourth reaction chamber where the fluid reaction temperature needed for the hybridization reaction is regulated by the temperature control system and the ExpressHyb hybridization solution (Clontech Laboratories, Inc., Mountain View, CA, USA), biotin-labeling mixture, wash buffer, blocking buffer, streptavidin conjugation, detection buffer, 3,3′-diaminobenzidine, and double-distilled water is delivered through the fluid delivery control system. After completion of the hybridization reaction, the chip color can be extracted and processed by the image system and image/information processing system.

Assessment of the sensitivity of the WEnCA-Chipball system

The detection limitation of the WEnCA-manual and WEnCA-Chipball systems were evaluated by a dilution test. Samples of 100, 25, 12, and 5 H358 lung cancer cells were added to 5 mL of peripheral blood obtained from a healthy volunteer. After the cells were added to the blood samples, either the WEnCA-manual or the WEnCA-Chipball system was used to perform the hybridization reaction by activating the *KRAS* Detection Chip. Next, the resultant reading was performed according to the description above, and the total score of the chip reaction was calculated.

Statistical analysis

All data were analyzed using the Statistical Package for Social Sciences version 11.5 (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to analyze the correlation between the Activating *KRAS* Detection

Chip results from both the WEnCA-manual and the WEnCA-Chipball system. Correlations where $p<0.05$ were considered statistically significant.

Results

Determination of the cutoff value of the Activating *KRAS* Detection Chip by the WEnCA-manual method

To determine the cutoff value of the Activating *KRAS* Detection Chip by the WEnCA method, we analyzed 200 cancer tissues of which 100 had the *KRAS* mutation and the others had wild-type *KRAS*. The 200 tissues collected underwent mRNA extraction and first cDNA labeling before reacting to the Activating *KRAS* Detection Chip by the WEnCA-manual method. After signal development, each gene spot density was normalized using the density of β-actin on the same chip. Next, the result obtained from the cancer tissue with *KRAS* mutation was divided by the normalized value obtained from the sample spot of the tissue without mutant *KRAS* to obtain the ratio. A ratio higher than 2 was defined as being positive for gene overexpression. In terms of analysis using WEnCA, to determine the weighted value of each gene spot, we divided the percentage of each gene overexpression in the 100 cancer tissues with the activating *KRAS* mutation to provide four classes (Figure 3). The gene spot that showed overexpression in over 80 cancer tissues had a weighted value of 4 (3 in 70–80 cancer tissues, 2 in 60–70 cancer tissues, and 1 in 50–60 cancer tissues). After the reaction through WEnCA, the positive gene spots were multiplied by their respective weighted values to obtain the total score of the chip. They then underwent analysis using the receiver operating characteristic curve with a positive reaction cutoff value of 20 (Figure 4). Results showed that the sensitivity reached 96% and the specificity reached 97%.

Detection limitation of the WEnCA-manual and WEnCA-Chipball assay

We evaluated the detection limitation of WEnCA-manual and WEnCA-Chipball systems, with the addition of 100, 25 and 12 cancer cells that possessed the activated mutant *KRAS* into 5 mL of blood. These gave total scores higher than the cutoff value of 20 in both systems, except when only six cells were added, in which case the total score equaled 8 in WEnCA-manual and 5 in the WEnCA-Chipball system (importantly, both lower than 20). No significant differences were found between the detection limitations of the two systems (Figure 5).

Clinical assessment of the accuracy of WEnCA-manual and WEnCA-Chipball assays

To establish the capabilities of the two systems for the clinical detection of *KRAS* activation from blood samples, we collected 209 samples of peripheral blood from pathologically proven lung cancer patients. All specimens were tested with the Activating *KRAS* Detection Chip by both the WEnCA-manual and WEnCA-Chipball methods. The paired cancer tissue with *KRAS* mutational status then served as the reference standard. The analytic results are shown in Table 1. There were 71 cancer tissues with *KRAS* mutation. Among them, 66 were positive through WEnCA-manual and 63 through WEnCA-Chipball. Moreover, among the 138 paired cancer tissues with wild-type *KRAS*, 130 were negative through both WEnCA-manual and WEnCA-Chipball assays. After statistical analysis, the sensitivity of the WEnCA-manual reached 93% and the specificity reached 94% (Table 2). On the other hand, the sensitivity through WEnCA-Chipball decreased to 89% and the specificity remained at 94%. Using WEnCA-Chipball, the average total score of the positive sample was 6.1 lower and the average total score of the negative sample was 3.9 lower. The overall average total score was 4.7 lower than the WEnCA-manual (Table 3).

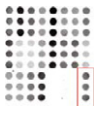
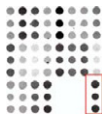
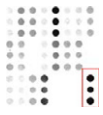
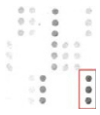
Cell numbers in 5 mL blood.	100	25	12	6
Array image				
WEnCA-manual	+ 42	+ 38	+ 30	– 8
WEnCA-Chipball	+ 37	+ 32	+ 26	– 5

Figure 5 Detection limitation of Weighted Enzymatic Chip array (WEnCA)-manual and WEnCA-Chipball systems.

Table 1 Mutant *KRAS* detected by Weighted Enzymatic Chip array (WEnCA)-manual and WEnCA-Chipball systems

WEnCA-manual	WEnCA-Chipball		
	Negative	Positive	Total
Negative	135	0	135
Positive	4	70	74
Total	139	70	209

Table 2 Sensitivity, specificity and accuracy of the Weighted Enzymatic Chip array (WEnCA)-Chipball and WEnCA-manual systems*

KRAS	WEnCA-Chipball (WEnCA-manual)		
	Negative	Positive	Total
Wild-type	130 (130)	8 (8)	138
Mutation	8 (5)	63 (66)	71
Total	138 (135)	71 (74)	209

*The sensitivity of WEnCA-manual was 93%, WEnCA-Chipball was 89%; specificity was 94% in both and the accuracy was 94% in the WEnCA-manual and 92% in the WEnCA-Chipball.

Table 3 Comparison of *KRAS* mutant detection analyzed by Weighted Enzymatic Chip array (WEnCA)-manual and WEnCA-Chipball systems

	Mean score		Difference (Chipball – Manual)
	WEnCA-manual	WEnCA-Chipball	
Positive specimens	46.1	40	−6.1
Negative specimens	13.8	9.9	−3.9
Total specimens	25.2	20.6	−4.6

Regarding operation time, the WEnCA-Chipball required only 7.5 hours whereas the WEnCA-manual required 3 days, approximately nine times the time required for the automated system. Regarding operational cost, the cost of the WEnCA-manual was US$80 per sample, including the administration fee, which was approximately five times more than that of WEnCA-Chipball.

Discussion

In recent years, target therapy has been extensively applied in addition to conventional chemotherapy and radiotherapy in the treatment of lung cancer, colorectal cancer, and breast cancer.[19–21] The *KRAS* mutations of cancer cells were determined using the membrane array technique which was established in our earlier research.[14] Direct detection of the overexpression of activated *KRAS* in peripheral blood is an indicator for target therapy such as cetuximab or gefitinib.[22] However, the highly technical criteria of chip operation and the complicated reading of the conventional method hinders its widespread availability to general clinics. Therefore, the current study successfully developed the WEnCA-Chipball to effectively solve those problems.

The results showed more consistent detection results compared with the original membrane array method while maintaining the same level of accuracy. In the WEnCA-Chipball system, the total operation time from input of samples to completion of the image analysis was about 7.5 hours, a substantial decrease in time compared with the 3 days required for the manually operated membrane array. In addition, human errors were substantially reduced. Evidently, the WEnCA-Chipball system not only provides an innovative automatic system for clinical target therapy efficacy evaluation, but also improves the clinical usability and accuracy compared with the manual method. Thus, our study shows that it is a practical means to assess the drug efficacy of clinical target treatment.

With regard to the application of fluid engineering techniques in the chip platform, the Lab-on-a-Chip,[23] developed by Micro-fluid Systems, is the most widely known. However, after 5 years of research, even though micro-fluidic chips have been extensively studied, the assessment of its clinical applications and the interpretation of results have not been possible; therefore, the clinical applications remain limited.[24,25] However, the WEnCA-Chipball system developed by this research team not only retains the advantages of the lab-on-a-chip device, but also overcomes the problem of the micro-fluidic chip's unsuitability for continuous operation and linkage to an interpretation system. As the world's first automatic chip analyzer, it will be useful in the future for the molecular diagnosis of infectious diseases, the detection of circulating tumor cell through chip replacements, or the assessment of drug efficacy.

In this study, we compared the total scores of activated *KRAS* expression of the same sample using both the Activating *KRAS* Detection Chip with WEnCA-manual and the WEnCA-Chipball. The results showed that the average score detected by WEnCA-Chipball was 4.7% lower than using WEnCA-manual. The decreased genetic expressions may be caused by the shortened hybridization time with the WEnCA-Chipball method, which was 1/15th of the manual operation time. In addition, among all of the tested samples, the sensitivity of the automatic system decreased by 4%. This was possibly due to the cutoff value used by WEnCA-Chipball being generated by the WEnCA-manual, which had a higher average detection score. Therefore, samples with scores distributed around the threshold value may be positive using the WEnCA-manual method, but negative using the WEnCA-Chipball method. To resolve this problem, an independent receiver operating characteristic curve can be precisely calculated by WEnCA-Chipball to obtain a lower cutoff value.

The WEnCA-Chipball system, through a built-in computer system, not only instantly produces the results of traditional chip analysis, but also connects to a global network. The detection results

and probe points can be transmitted across the world using common software. All the probe networks around the world can be completed through the prevalent Chipball-WEnCA system. Our study shows that the Chipball-WEnCA system can have extensive applications in clinical medicine and holds great potential for future development.

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References

1. Yu M, Wan Y, Zou Q. Prognostic significance of BP1 mRNA expression level in patients with non-small cell lung cancer. *Clin Biochem* 2008;41:824–30.
2. Wu CH, Lin SR, Yu FJ, et al. Development of a high-throughput membrane-array method for molecular diagnosis of circulating tumor cells in patients with gastric cancers. *Int J Cancer* 2006;119:373–9.
3. Poulsen TT, Pedersen N, Perin MS, et al. Specific sensitivity of small cell lung cancer cell lines to the snake venom toxin taipoxin. *Lung Cancer* 2005;50:329–37.
4. Wu CH, Lin SR, Hsieh JS, et al. Molecular detection of disseminated tumor cells in the peripheral blood of patients with gastric cancer: evaluation of their prognostic significance. *Dis Markers* 2006;22:103–9.
5. Saito D, Coutinho LL, Borges Saito CP, et al. Real-time polymerase chain reaction quantification of *Porphyromonas gingivalis* and *Tannerella forsythia* in primary endodontic infections. *J Endod* 2009;35:1518–24.
6. Yang M, Cheng A, Wang M, et al. Development and application of a one-step real-time TaqMan RT-PCR assay for detection of Duck hepatitis virus type 1. *J Virol Methods* 2008;153:55–60.
7. Karakoc AE, Berkem R, Beyaz E. Detection of hepatitis B virus deoxyribonucleic acid using real-time polymerase chain reaction method in pooled-plasma samples of Turkish blood donors. *Clin Lab* 2009;55:229–34.
8. Böhl M, Schwenzer B. A potent inhibitor of prothrombin gene expression as a result of standardized target site selection and design of antisense oligonucleotides. *Oligonucleotides* 2005;15:172–82.
9. Ball TB, Plummer FA, HayGlass KT. Improved mRNA quantitation in LightCycler RT-PCR. *Int Arch Allergy Immunol* 2003;130:82–6.
10. Walder RY, Hayes JR, Walder JA. Use of PCR primers containing a 3′-terminal ribose residue to prevent cross-contamination of amplified sequences. *Nucleic Acids Res* 1993;21:4339–43.
11. Popovtzer R, Neufeld T, Popovtzer A, et al. Electrochemical lab on a chip for high-throughput analysis of anticancer drugs efficiency. *Nanomedicine* 2008;4:121–6.
12. Chen CC, Hou MF, Wang JY, et al. Simultaneous detection of multiple mRNA markers CK19, CEA, c-Met, Her2/neu and hMAM with membrane array, an innovative technique with a great potential for breast cancer diagnosis. *Cancer Lett* 2006;240:279–88.
13. Chen CC, Chang TW, Chen FM, et al. Combination of multiple mRNA markers (PTTG1, Survivin, UbcH10 and TK1) in diagnosis of Taiwanese patients with breast cancer by membrane array. *Oncology* 2006;70:438–46.
14. Tsao DA, Yang MJ, Chang HJ, et al. A fast and convenient new technique to detect the therapeutic target, K-ras mutant, from peripheral blood in non-small cell lung cancer patients. *Lung Cancer* 2009 (In press).
15. Matsugi J, Murao K. Study on construction of a cDNA library corresponding to an amino acid-specific tRNA and influence of the modified nucleotide upon nucleotide misincorporations in reverse transcription. *Biochim Biophys Acta* 2001;1521:81–8.
16. Zhu Q, von Dippe P, Xing W, et al. Membrane topology and cell surface targeting of microsomal epoxide hydrolase: evidence for multiple topological orientations. *J Biol Chem* 1999;274:27898–904.
17. Harder N, Mora-Bermúdez F, Godinez WJ, et al. Automatic analysis of dividing cells in live cell movies to detect mitotic delays and correlate phenotypes in time. *Genome Res* 2009;19:2113–24.
18. Sajda P. Machine learning for detection and diagnosis of disease. *Annu Rev Biomed Eng* 2006;8:537–65.
19. Sobrero A, Aschele C, Guglielmi A, et al. Resistance to 5-fluorouracil and 5-fluoro-2′-deoxyuridine mechanisms and clinical implications. *J Chemother* 1990;2(Suppl 1):S12–6.
20. Rossi A, Maione P, Palazzolo G, et al. New targeted therapies and small-cell lung cancer. *Clin Lung Cancer* 2008;9:271–9.
21. Wu JT, Kral JG. The NF-κB/IκB signaling system: a molecular target in breast cancer therapy. *J Surg Res* 2005;123:158–69.
22. Modjtahedi H, Essapen S. Epidermal growth factor receptor inhibitors in cancer treatment: advances, challenges and opportunities. *Anticancer Drugs* 2009;20:851–5.
23. Funfak A, Brösing A, Brand M, et al. Micro fluid segment technique for screening and development studies on Danio rerio embryos. *Lab Chip* 2007;7:1132–8.
24. Duenwald S, Zhou M, Wang Y, et al. Development of a microarray platform for FFPET profiling: application to the classification of human tumors. *J Transl Med* 2009;7:65.
25. Ohno K, Tachikawa K, Manz A. Microfluidics: applications for analytical purposes in chemistry and biochemistry. *Electrophoresis* 2008;29:4443–53.